

# Characterization of early induced genes in *Arabidopsis thaliana* responding to bacterial inoculation: identification of centrin and of a novel protein with two regions related to kinase domains

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**Abstract** The early response to bacterial inoculation has been investigated and two *Arabidopsis* genes, *ap3.3a* and *ap4.3a* have been characterized. The AP3.3A protein showed high identity to centrin, a ubiquitous cytoskeletal protein first identified in unicellular green alga. Amino-acid sequence analyses of the AP4.3A protein indicates that the second gene characterized encodes an unusual protein with two putative kinase domains. Expression of *ap3.3a* and *ap4.3a* was rapidly induced after pathogen inoculation. A role of *ap3.3a* in plant defense could be postulated based on its preferential induction during the incompatible interactions analyzed. In contrast, activation of *ap4.3a* was not specific and could be related to a more general stress response.

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**Key words:** Bacterial infection; Differentially induced gene; Centrin; Kinase; *Arabidopsis thaliana*

## 1. Introduction

Plants have co-evolved with their pathogens by developing mechanisms to protect themselves from microbial attack. The perception of a potential pathogen is transduced to intracellular responses by specific signalling pathways triggered at the infected areas. Localized cell death (hypersensitive reaction) often occurs at the sites of pathogen ingress and it is frequently correlated with plant defense [1,2]. In addition to this, the synthesis of a battery of defense-related products, such as callose and lignin deposits, antimicrobial compounds (phytoalexins), hydrolytic enzymes and antimicrobial proteins, is known to contribute to limit pathogen growth and host colonization [3]. The local response is also involved in triggering a subsequent non-specific resistance that acts throughout the entire plant. This phenomenon, referred to as systemic response, is correlated with the induction of a set of defense-response genes in uninfected leaves that protects the potentially weakened tissues from secondary infections [4].

A general understanding of the events mediating the plant defense response is presently emerging, however, the molecular basis of pathogen recognition and early signalling leading to activation of defense responses are largely unknown [5]. Many of these responses are due to transcriptional induction of specific defense-related genes. In contrast, several processes are known to occur by activation of preexisting components. An example of the latter are the transient changes in the

permeability of the plasma membrane and the release of active oxygen species such as  $O_2^-$  and  $H_2O_2$ , referred to as oxidative burst, which have often been detected as the first responses to pathogen perception [6–9]. Activation of preexisting kinases and phosphatases is considered to be the most likely downstream steps to the above described mechanisms. Several groups have recently identified distinct MAP kinases that are rapidly activated in response to external stimuli including salicylic acid, fungal elicitor, and wounding [10–12]. In addition, transcriptional induction of specific kinases have also been found to occur in response to pathogen infection as well as other plant treatments related to the defense response [13,14].

We have used differential display [15] to identify plant genes whose expression was early induced in *Arabidopsis* leaves after inoculation with an incompatible strain of *Xanthomonas campestris* pv. *campestris*. Changes in gene expression were examined 1 h after bacterial inoculation and two transcripts, coding for a calcium binding protein (centrin) and a novel putative protein kinase, respectively, were characterized.

## 2. Materials and methods

### 2.1. Plant material

*Arabidopsis thaliana* plants ecotype Columbia (Col-0) were grown in chamber (22°, 70% relative humidity,  $150 \mu E m^{-2} s^{-1}$  fluorescent illumination) under a 14-h light/10-h dark photoperiod. Plants were treated and examined between 4 and 5 weeks after seed germination.

### 2.2. Bacterial strains

Virulent bacteria, *Xanthomonas campestris* pv. *campestris* 8004 and *Pseudomonas syringae* pv. *tomato* DC3000, and avirulent strains *Xanthomonas campestris* pv. *campestris* 147 and *Pseudomonas syringae* pv. *tomato* DC3000 *avrRpm1* were used. Bacterial inoculation was done by injection of the leaves with a late-logarithmic culture using a syringe. *Xanthomonas* strains were grown in Kado medium (1% yeast extract, 0.8% casamino acids, 1% saccharose, 6.5 mM  $K_2HPO_4$  and 6 mM  $MgSO_4 \cdot 7H_2O$ ) at 27°C. *Pseudomonas* strains were grown in King's medium (2% proteose peptone, 2% glycerol, 6.5 mM  $K_2HPO_4$  and 6 mM  $MgSO_4 \cdot 7H_2O$ ) at 27°C. After centrifugation, pellets were resuspended in sterile water to reach a final concentration of  $10^7$  bacteria/ml. Plants were infected by forcing bacterial suspension through the stomata by gently pressing the syringe against the abaxial side of the leaves. Water inoculated plants were used as control.

### 2.3. Recombinant DNA techniques

DNA techniques were carried out according to standard protocols [16,17]. Plasmid vectors pT3T7 and pBluescript were used for DNA cloning. DNA sequence was determined by using the chain-termination method [18]. Oligonucleotides were synthesized using an Applied Biosystems 380B DNA synthesizer. Sequences were analyzed using the GCG package (version 9.0, Genetics Computer Group, Madison, WI, USA). Database searches involved BLAST [19] and FASTA [20].

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#### 2.4. Differential display

Differential display analyses [15] were carried out on total RNA extracted from *Arabidopsis* leaves 1 h after inoculation with *Xanthomonas campestris* pv. *campestris* (strain 147). In addition, RNA from healthy untreated leaves was extracted and used as a control. RNA was extracted from leaf tissue [21]. Independently prepared RNA samples were subjected to this analysis to identify cDNA bands that were reproducibly induced in response to bacterial inoculation. After DNase treatment, 2 µg of each total RNA sample were used for reverse transcription and subsequent polymerase chain reaction (PCR) by using an RNAmapping kit (GenHunter, Brookline, MA, USA). Each RNA sample was reverse transcribed using T12MC followed by PCR amplification in the presence of T12MC and any of each of the five arbitrary 10-mers, AP-1, AP-2, AP-3, AP-4, and AP-5, provided with the kit. Reaction products were separated on 6% sequencing gels and visualized by autoradiography. Selected bands were excised and re-amplified according to the manufacturer's protocol. Reamplified products were purified from an agarose gel, cloned into the pT3T7 vector (Stratagene), sequenced and used as probes for RNA blot analysis and cDNA library screening. cDNA fragments, *AP3.3A* and *AP4.3A*, characterized here were amplified by using T12MC and primers AP-3 (5'-AGGTGACCGT-3') or AP-4 (5'-GGTACTCCAC-3'), respectively.

#### 2.5. cDNA library construction

Polyadenylated (poly(A)<sup>+</sup>) mRNA was prepared from total RNA extracted from *Arabidopsis* leaves 1 h after inoculation with *Xanthomonas campestris* pv. *campestris* (strain 147). Complementary DNA was synthesized and cloned into the Lambda ZAP II vector (Stratagene) according to the manufacturer's instructions.

#### 2.6. Screening of the *A. thaliana* library

Recombinant phages were propagated in *E. coli* XL-1 Blue and screened by plaque hybridization with radioactive riboprobes generated using the Stratagene RNA transcription kit. Filters (Hybond-N; Amersham) were hybridized overnight at 42°C in 50% formamide, 5×SSC, 5×Denhardt's solution, 0.5% SDS and 20 µg/ml denatured herring sperm DNA, and washed twice with 5×SSC, 0.1% SDS at 42°C for 30 min and then twice with 2×SSC, 0.1% SDS at 42°C for 30 min. Filters were exposed to Hyperfilm MPfilm (Amersham) with an intensifying screen at -80°C for 24 h. Positives clones were excised with the helper phage and recircularized to generate a subclone in the

pBluescript SK<sup>-</sup> phagemid vector (Stratagene). A Col-0 *Arabidopsis* genomic library from Mulligan and Davis, constructed in λ GEM11 (Promega), was used for isolation of the *AP4.3A* genomic clone. At least 100 000 plaques were screened by plaque hybridization using an *ap4.3a* cDNA as a probe. Hybridization and filter washes were carried out as described above.

#### 2.7. Analysis of gene expression

Gene expression was examined in response to bacterial inoculation as well as to inoculation with water, salicylic acid (1 mM) or jasmonic acid (50 µM). Treatments were carried out by injection of the leaves using a syringe. In addition, leaves were wounded by gently rubbing of the upper epidermis with wet Carborundum. Tissues for RNA extraction were harvested at the indicated times and immediately frozen in liquid nitrogen prior to RNA extraction [22]. Total RNA (5 µg per lane) was electrophoresed in 1.5% agarose-formaldehyde gels [17] and transferred to Hybond-N membranes (Amersham). The amount of loaded RNA was verified by addition of ethidium bromide to the samples and photography under UV light after electrophoresis. Single-stranded riboprobes derived from the *Arabidopsis* cDNAs characterized were generated using the Stratagene RNA transcription kit. In addition, riboprobes were prepared from an *Arabidopsis PR-1* clone [23] and a *Vsp* (vegetative storage protein) encoding cDNA (EST accession number T22506). Blots were hybridized at 68°C in 50% formamide, 5×SSC, 5×Denhardt's solution, 0.5% SDS, and 20 µg/ml denatured herring sperm DNA. Hybridizations were washed at 68°C twice with 5×SSC, 0.1% SDS for 30 min, twice with 1×SSC, 0.1% SDS for 30 min.

### 3. Results

#### 3.1. Identification of early induced genes in response to bacterial inoculation

We used differential mRNA display [15] to identify new genes which are early induced during bacterial infection in *Arabidopsis thaliana*. To this end, total RNA was extracted from leaves 1 h after inoculation with the bacterium *Xanthomonas campestris* pv. *campestris* (strain 147) previously characterized [24]. In addition, we included as a control RNA extracted from healthy untreated leaves.

Differences in gene expression were examined by analysis of two separated differential display experiments. cDNA fragments shown to be reproducibly induced after bacterial treatment were characterized by sequencing and expression analysis (data not shown). Based on these studies, two cDNA fragments designated *AP3.3A* and *AP4.3A* (Fig. 1), were selected for further characterization.

As described in Section 2, a cDNA library was prepared with total RNA extracted from *Arabidopsis* leaves 1 h after bacterial inoculation, and screened using *AP3.3A* and *AP4.3A* derived probes. A 719-bp cDNA fragment hybridizing to *AP3.3A* was isolated and examined by sequencing. On the other hand, four positive clones were isolated when using the *AP4.3A* fragment. The longest cDNA (967 bp) was sequenced completely. In both cases, the cDNAs examined were identical, within their overlapping regions, to the DNA fragments obtained by differential display.

#### 3.2. The *ap3.3a* cDNA encodes a protein with homology to centrin

The complete DNA sequence of *ap3.3a* is shown in Fig. 2. The *ap3.3a* cDNA insert was 719 bp long and its larger open reading frame (504 nt) encodes a polypeptide of 168 amino acids with a predicted molecular weight of approximately 20 000. A stop codon in-frame with the proposed coding sequence is present in the 5' end of the cDNA at 64 nucleotides upstream from the first ATG of the reading frame. The pu-

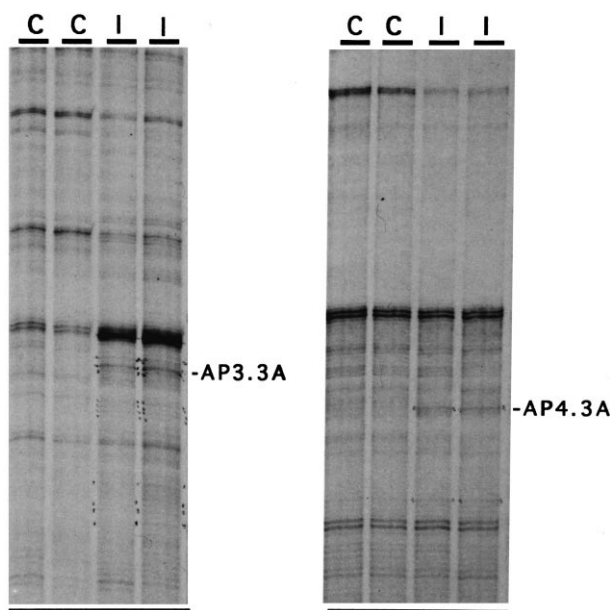


Fig. 1. Differential display analysis of total RNA isolated from *Arabidopsis* leaves 1 h after inoculation with *Xanthomonas campestris* pv. *campestris* (147) (I) or control untreated leaves (C). Duplicated samples were processed in parallel. Positions of the *AP3.3A* and *AP4.3A* DNA fragments characterized are indicated.

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-66 TAGATTTCCTTTAGACGCGCAAACTGAAAGAGCTTTTGATCGATCGATTGGAGGATCCGTA
-6 GAAGATATGTCGAGTATATACAGAAGCTTTTCGAGAAAAGAGAAACCGAGACGTCACTAT
    M S S I Y R T V S R K E K P R R H H 18
56 GGATTGACTACACAGAAGAACGAAGAGATTAAAGGAGCTTTTGAGCTATTTGACACTGAT
    G L T T Q K K Q E I K E A F E L F D T D 38
116 GGTTCCTGGTACCATTTGATGCTTAAAGAGCTTTAATGTTGCTATAGAGGGCGCTTGGTTTGA
    G S G T T I D A K E L N V A M R A L G F E 58
176 ATGACGGAAGAGCAAACTCAACAAAATGATAGCTGATGATGGATAAAGATGGAAGTGGAGCT
    M T E E Q I N K M I A D V D K D G S G A 78
236 ATAGATTTTTGATGAGTTTGTTCATATGATGACTGCTAAGATTGGTGAAGAGACACAAA
    I D F D E F V H M M T A K I G E R D T K 98
296 GAAGAGCTCACTAAAGCATTCAGATCATGATCTTGACAAAATGGGAAGATATCTCCG
    E E L T K A F Q I I D L D K N G K I T S P 118
356 GATGATATCAAAACGATGGCAAGAGCTTGGGTGAGAAITTCACATGATGCTGAGATACGA
    D D I K R M A K D L G E N F T D A E I R 138
416 GAGATGTTTGAAGAAGCAGACCGGACCGTGATGGTGAAGTTAACATGGATGAATTCATG
    E M V E E A D R D R D G E V N M D E F M 158
476 AGGATGATGAGGAGAACTGCTTATGGTGTAACTAGAAAGGTTGTTGCTGTTCTTAACGA
    R M M R R T A Y G G N 169
536 TATTTGATGAGCTCTATATATGTTCTTAACTGAGGCTTAATATCTGTAATATTATTAC
596 TGAAGATACATATACACAGTTTAACTATAGCTCAGCTTAATGCGCAAAAAA

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Fig. 2. Nucleotide and predicted amino acid sequence of the *ap3.3a* cDNA (GenBank accession no. AJ009672). Nucleotides are numbered with the first nucleotide of the initiating ATG designated as +1. Amino acid sequence is numbered on the right. Amino acid residues proposed to be involved in  $\text{Ca}^{2+}$  binding are underlined.

tative amino acid sequence of AP3.3A shows high similarity to centrin, an ubiquitous cytoskeletal protein first identified in the unicellular green alga *Chlamydomonas reinhardtii* [25]. Centrin has been hypothesized to have a three-dimensional configuration that forms four EF-hand structures involved in  $\text{Ca}^{2+}$  binding [26]. Amino acid residues predicted to be involved in  $\text{Ca}^{2+}$  binding were found to be conserved in AP3.3A (Fig. 2). The sequence identity between AP3.3A and other known centrin proteins ranged from 79%, with that of the land plant *Atriplex nummularia* [27], to 52% with human centrin [28]. A 65% identity was found between AP3.3A and the protein encoded by the *Arabidopsis* locus ATAP21 (ESSA, EU *Arabidopsis* sequencing project; accession no. Z99707). Sequence alignment of AP3.3A with that of centrins is shown in Fig. 3. The sequence similarity revealed by these studies showed that *ap3.3a* encodes a centrin protein and that the cDNA isolated here corresponds to a different protein to that encoded by the *Arabidopsis* clone Z99707.

### 3.3. The *ap4.3a* gene encodes a putative protein kinase

A genomic library was screened using an *ap4.3a* probe derived from the previously selected 967-bp cDNA and a 6.5-kb genomic fragment was isolated. Sequence analysis revealed that the genomic clone identified corresponded to the cDNA fragment used for the screening and that it contained an interrupted open reading frame of 2727 nt encoding a polypeptide of 909 amino acids with a predicted molecular weight of approximately 110 000. A stop codon in-frame with the suggested coding region was found at 100 bp upstream from the first ATG. The complete DNA sequence of *ap4.3a* and its encoded protein is shown in Fig. 4. The putative amino acid sequence of AP4.3A shows significant homology to protein kinases and two different protein regions spanning residues 174–455 and 590–852, respectively, could be defined as putative kinase domains. A schematic representation of the AP4.3A protein is shown in Fig. 5. The AP4.3A protein region corresponding to amino acids 174–455, designated as kinase domain A, shares a 30–38% identity with receptor like protein kinases from different plants [29,30]. Similarly, the second putative kinase domain, designated as B, shows homology to the same type of proteins, although the level of identity identified was reduced to 25–30% [29,30]. Homology of AP4.3A was essentially restricted to the kinase domain of the different receptor like proteins examined and no signifi-

cant homology was observed with sequences located outside this region. Further analysis of domains A and B allows to recognize the XI subdomains characteristic of functional kinases [31], although some residues essential for activity are lacking in the B domain. The AP4.3A regions falling outside of these domains did not show significant homology with other protein sequences deposited in the data base banks. In addition to published sequences, the AP4.3A protein shows a 99% identity with the unpublished sequence (accession no. AC003974) of a putative kinase protein present on chromosome II (BAC F24L7).

### 3.4. Expression of *ap3.3a* is early induced in response to pathogen infection

The expression of *ap3.3a* was investigated at different time intervals in response to inoculation with two *Xanthomonas* strains, *X. c. pv. campestris* (147) and *X. c. pv. campestris* (8004) [32] that establish an incompatible and a compatible interaction, respectively with *Arabidopsis* ecotype Col-0. In addition, gene expression was examined in response to inoculation with the virulent strain *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 and the avirulent bacterium *Pst* DC3000 *avrRpm1*. It has been shown that *Arabidopsis* ecotype Col-0 is susceptible to *Pst* strain DC3000 [33] but resistant to the same strain carrying the avirulence gene *avrRpm1* [34,35]. The pathosystems described allowed us to examine whether gene expression was induced in response to bacterial pathogen infection and to determine whether or not gene induction was specific for a particular type of interaction.

As shown in Fig. 6, no *ap3.3a* transcripts were detected in

AP33A	MSSIYRTV- - -SRKEKPR- -RHHGLTQKK	25
A.nummularia	MSSA-IRTV- - -RKDKPR-GRHGLTQQR	24
A.thaliana	MSEAAQ- L- - -RRGLKPK- GKTYGLTQKR	25
C.reinhardtii	MSYKAKT- - -VSSARRDQK- - -GRVLTEECK	27
Human	MASGFKKPSAASTGQKRVKVPKPELTEDCK	30
AP33A	QEI KEAFELFDTDGSGTI DAKELNVAMRAL	55
A.nummularia	QEI KEAFELFDTDGSGTI DAKELNVAMRAL	55
A.thaliana	REI RE- -FDLFD- -DGSGSI DASELVAMRSL	55
C.reinhardtii	QEI REAFDLFDTDGSGTI DAKELNVAMRAL	57
Human	QEVREAFDLFDVDSGSI DAKELNVAMRAL	60
AP33A	GFEMTEEQI NKMI ADVDKDGSGL DFDEFV	85
A.nummularia	GFEMTEEQI NKMI ADVDKDGSGL DFDEFV	85
A.thaliana	GFEMNNQNI NELMAVDKNGSGAI DFDEFV	84
C.reinhardtii	GFEPKKEEL KMI SEIDKDGSGTI DFEEFL	87
Human	GFEPKKEEMKKMI SEVDRE- - -TKLSENDEL	90
AP33A	HMMTAKI GERDTKEELTKAFQI I DLKNGK	115
A.nummularia	HMMTAKI GERDTKEELTKAFRI I DDNNNGK	114
A.thaliana	HMMTTKGERDSI DELSKAFKI I DDNNNGK	115
C.reinhardtii	TMMTAKVGERDSREEL LKAFRLFDNNSGT	117
Human	AVMTAKMSEKDTKEEL LKAFRLFDDETCK	120
AP33A	I SPDDI KRMADLGENFTDAE I REMVEEAD	145
A.nummularia	I SPEDI QR I AKEL GENFTVKDI QDMI EEEAD	144
A.thaliana	I SPDDI KMI AKEL GENFTNDI EEMI EEEAD	145
C.reinhardtii	I TI KDLRRVAKEL GENLTFEEL QEMI AEAD	147
Human	I SFKNLKRVA NEL GENLTFEEL QEMI DEAD	150
AP33A	RDRDGEVNMDFE FMRMMRRRTAYGGN	169
A.nummularia	RDRDGEVNVEE FLMKMKRTSYA- Y	167
A.thaliana	RDKDGEVN- EEFMKMKRTSYG	167
C.reinhardtii	RNDDEI DEDEFI RIMKKISL- - F	169
Human	RDKDGEVNEEFEL RIMKKISL- - Y	172

Fig. 3. Sequence alignment of *Arabidopsis* AP3.3A. Regions of identity (black) have been highlighted. Alignment and similarity identification were performed using the MegAlign program. Sequences shown correspond to AP3.3A and centrins from *Atriplex nummularia* [26], *Arabidopsis thaliana* locus ATAP21 (ESSA, EU *Arabidopsis* sequencing project; accession no. Z99707), *Chlamydomonas reinhardtii* [25] and human [27].

[illegible]

Fig. 4. Nucleotide and predicted amino acid sequence of *AP4.3A* (GenBank accession no. AJ009671). Nucleotides are numbered with the first nucleotide of the initiating ATG designated as +1. Amino acids are numbered on the right.

control untreated leaves while *ap3.3a* hybridized to a single transcript of approximately 700 bp (corresponding to the size predicted for the *ap3.3a* mRNA) in RNA samples extracted 1 h after *X. c. pv. campestris* (147) treatment, and 4 h after

inoculation with the avirulent strain *Pst* DC3000 *avrRpm1*. An additional RNA transcript of approximately 300 bp was detected when hybridizations were carried out at lower stringency conditions (data not shown). Currently, it is not known whether this small RNA transcript corresponds to a distinct mRNA or it represents a processed form of the *ap3.3a* mRNA. The detection of centrin-related transcripts resulting from RNA processing events has been previously reported in *Atriplex nummularia* [36].

In contrast to the early activation of gene expression observed after inoculation with the avirulent strains, induction of *ap3.3a* was significantly delayed in plants responding to inoculation with the two compatible bacteria examined. A weak induction was observed 24 h after inoculation with *X. c. pv. campestris* 8004 whereas *ap3.3a* transcripts were barely detected 48 h after *Pst* DC3000 inoculation. No activation of gene expression was observed in water treated tissues.

### 3.5. Expression of *ap4.3a* is early activated in a non-specific manner

The expression of *ap4.3a* was examined in response to inoculation with the bacterial strains described above. As shown in Fig. 7, expression of the *ap4.3a* gene was found to be induced in response to bacterial inoculation. However, induction of gene expression was not observed to be specifically induced in response to a particular strain. A single transcript of approximately 3 kb, corresponding to the size predicted for the *ap4.3a* mRNA, was found to accumulate 15 min after inoculation with both incompatible or compatible bacteria. Furthermore, *ap4.3a* mRNA followed a similar pattern of accumulation in response to the different bacteria examined decreasing to almost undetectable levels in RNA samples extracted 2 h after inoculation.

### 3.6. Analysis of gene expression in response to signal molecules mediating the plant response to pathogen infection

The role of salicylic acid (SA) and jasmonic acid (JA) as cellular signals, affecting the plant response to pathogen infection, has been documented [4,37]. To evaluate whether these cellular signals had any effect on the expression of *ap3.3a* and *ap4.3a*, the level of transcripts in leaves of *Arabidopsis* plants was examined after exogenous application of SA (1 mM) or JA (50  $\mu$ M). As shown in Fig. 8, no induction of *ap3.3a* expression was observed in response to any of the two treatments examined, whereas expression of *ap4.3a* was weakly induced in response to SA and JA. *ap4.3a* transcripts were detected 30 min after SA injection declining to undetectable levels 1 h after inoculation. Activation of gene expression by JA was slightly delayed and a weak hybridization signal was observed in RNA samples extracted 1 h after treatment. High levels of transcripts were observed when expression of *PR-1* and *Vsp*, used as a control for treatments with SA and JA, respectively, was examined (Fig. 8C).

In addition to pathogen infection, JA is known to play an important role in the signalling pathway controlling the plant response to mechanical stress [38]. Analyses of gene induction by wounding revealed that *ap3.3a* expression was activated 15 min after mechanical damage. The level of RNA was maintained for the first 30 min and decreased to undetectable levels in plants examined 2 h after treatment. The level of *ap4.3a* RNA increased 1 h after mechanical wounding and was maintained during the first 2 h after treatment.

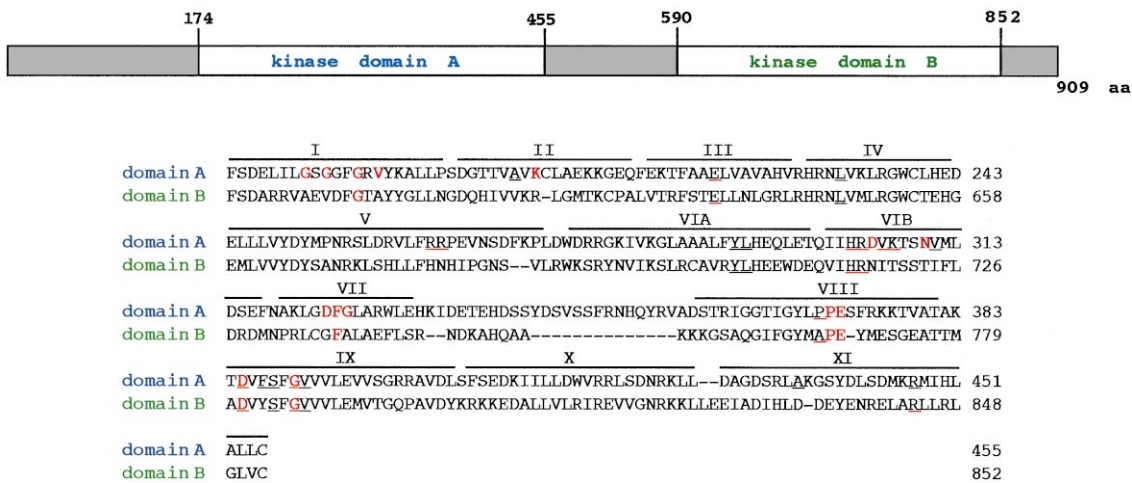


Fig. 5. Schematic representation of the AP4.3A predicted protein. The sequence corresponding to two putative kinase domains A and B is shown. Invariant amino acids essential for maximal enzyme activity are shown in red. Nearly invariant residues are underlined in red and highly conserved amino acids are underlined in black. Roman numbers indicate subdomains characteristic of functional kinases. Hyphens denote gaps introduced to optimize the alignment.

4. Discussion

The early response of *Arabidopsis thaliana* to bacterial infection has been examined and two cDNAs have been identified. *ap3.3a* shows homology to centrin, a cytoskeleton calcium-binding protein found to be associated with centrioles/basal bodies, centrosomes, and mitotic spindle poles in cells of diverse evolutionary lineages, including algal, higher plants, invertebrates, and mammalian cells [25,27,28]. Expression of

*ap3.3a* was found to be induced in a specific manner, being preferentially activated by inoculation with avirulent bacteria. This specificity was more evident in plants responding to inoculation with the compatible strain *Pst* DC3000, where the level of transcripts accumulated was significantly reduced with respect to that detected by inoculation with the avirulent strain *Pst* DC3000 *avrRpm1*. Early activation of gene expression in response to avirulent pathogens has been characterized for various genes involved in plant defense responses [39]. In

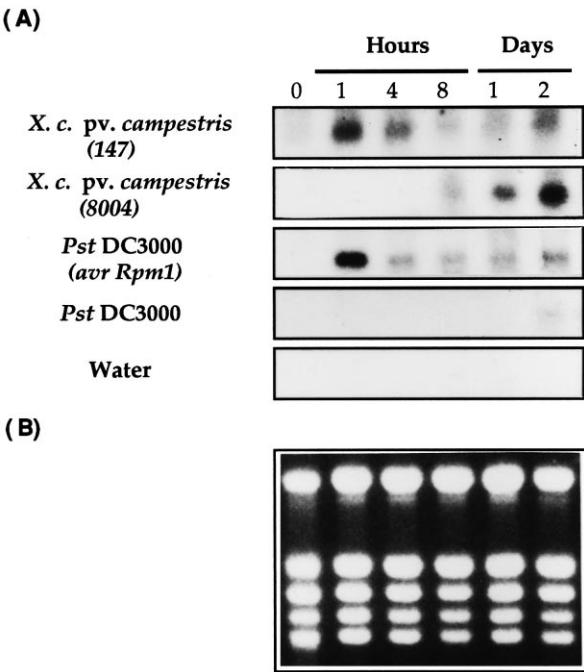


Fig. 6. Specificity of *ap3.3a* induction in response to bacterial infection. A: RNA was extracted from *Arabidopsis* leaves in response to inoculation with *Xanthomonas campestris* pv. *campestris* (147), *Xanthomonas campestris* pv. *campestris* (8004), *Pseudomonas syringae* pv. *tomato* DC3000, or *P.s.* pv. *tomato* DC3000 *avrRPM1*. Inoculation with water was used as a control. B: Ethidium bromide staining used as a control for equal loading of RNA is shown.

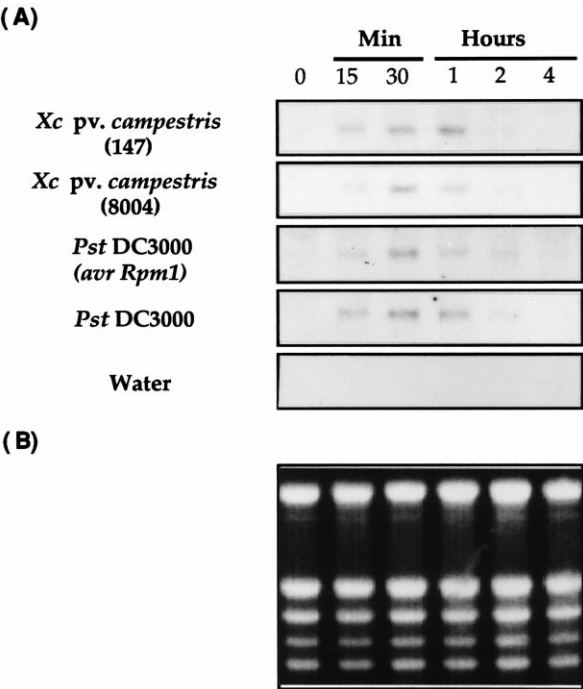


Fig. 7. Analysis of *ap4.3a* expression in response to bacterial infection. A: RNA was extracted from *Arabidopsis* leaves in response to inoculation with *Xanthomonas campestris* pv. *campestris* (147), *Xanthomonas campestris* pv. *campestris* (8004), *Pseudomonas syringae* pv. *tomato* DC3000, or *P.s.* pv. *tomato* DC3000 *avrRPM1*. Inoculation with water was used as a control. B: Ethidium bromide staining used as a control for equal loading of RNA is shown.



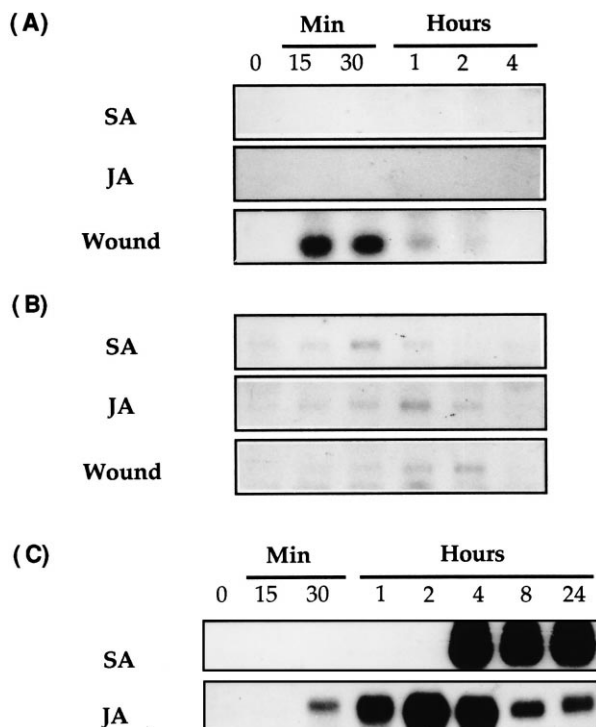


Fig. 8. Analysis of *ap3.3a* and *ap4.3a* expression in response to cellular signals mediating the plant response to pathogen infection. RNA was extracted at different time intervals in response to salicylic acid (SA), jasmonic acid (JA) or wounding. A: Blots were hybridized to riboprobes derived from the *ap3.3a* cDNA. B: Blots were hybridized to riboprobes derived from the *ap4.3a* gene. C: RNA samples extracted after SA treatment were hybridized to riboprobes derived from a PR-1 cDNA. RNA samples prepared after JA inoculation were hybridized to a *Vsp* encoding cDNA.

this scenario, the differences in the timing of *ap3.3a* induction observed between the two incompatible and the compatible interactions examined, could be indicative of a role in plant defense. We found that, in addition to bacterial treatment, the expression of *ap3.3a* was also induced by wounding, whereas no induction was observed in response to treatment with SA and JA. These results could indicate that activation of *ap3.3a* occurs through an SA- or JA-independent signalling pathway. Alternatively, it is possible that gene expression would be triggered by cellular signals generated prior to the increase of SA and JA in the plant tissues. In accordance with the last suggestion, expression of *ap3.3a* was rapidly activated after bacterial inoculation and wounding. On the other hand, the fact that centrin is a calcium-binding protein [40] and that  $\text{Ca}^{2+}$  plays an important role as an early signal in responses such as pathogen attack and mechanical damage [8,41], suggests that the induction of *ap3.3a* might be related to the elevation of cytoplasmic calcium which is involved in the early signal transduction leading to a HR [42].

In animal and algal cells, centrin has been found to be associated with the cytoskeleton, and a role in microtubule severing and cytoskeleton reorganization has been shown [43]. Immunolocalization analyses in a number of higher plants identified the presence of centrin at various cellular locations, including the microtubules [44], and a possible role of centrin in mediating developmental and environmental signals such as touch and heat has been suggested [36]. Ac-

cording to our results, an additional role of centrin in the response to pathogen infection and wounding can be envisaged. One can speculate that centrin could perform a function related to intracellular reorganization during early infection. This suggestion is based on results showing that infection of parsley cells with *Phytophthora infestans* is associated with a rapid translocation of cytoplasm and nucleus to the fungal penetration site, a response that is mediated by depolymerization of the microtubular network [45]. Along the same line, the arrangement of microtubules and microfilaments was found to play an important role in the expression of non-host resistance in barley [46].

As for *ap3.3a*, activation of *ap4.3a* was found to occur rapidly in response to bacterial infection and wounding. Furthermore, expression was also induced by SA and JA treatment. Expression of *ap4.3a* was not specifically induced in response to a particular type of pathogen but activation of gene expression followed a similar pattern of mRNA accumulation in all the plant-pathogen interactions examined. These results disfavor a role of AP4.3A in plant defense but a more general function in response to stress could be postulated. Sequence analysis of the *ap4.3a* encoded protein revealed the presence of two amino acid stretches, designated as A and B, related to kinase domains. Despite the absence of specific amino acids required for enzyme activity within the B domain, the presence of all the subdomains characteristic of complete kinases in the A domain allows to suggest that the AP4.3A protein could act as a functional kinase. Proteins containing two kinase domains have been previously identified in *Dictyostelium discoideum* and vertebrates where, interestingly, one of the two domains is often truncated [47,48]. In vertebrates, these proteins are designated as Janus kinases (JAKs) and have been found to be associated to transmembrane receptors that mediate cellular responses to external stimuli. Aggregation of receptors, after binding to the corresponding ligand, results in their association with JAKs and activation of downstream signalling processes. Whether AP4.3A could function in a similar manner in plant signal transduction is presently unknown. The rapid activation of gene expression together with a putative kinase activity could be correlated with the early phosphorylating events associated with kinases and signal transduction.

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